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Reactivities of Human Sera with Human Herpesvirus-8-Infected BCBL-1 Cells and Identification of HHV-8-Specific Proteins and Glycoproteins and the Encoding cDNAs

Bala Chandran,*[1] Marilyn S. Smith,*,† David M. Koelle,‡,§ Lawrence Corey,‡,§ Rebecca Horvat,¶ and Elliot Goldstein*,†

*Department of Microbiology, Molecular Genetics and Immunology, †Division of Infectious Diseases, Department of Medicine, ¶Department of Pathology and Clinical Laboratories, The University of Kansas Medical Center, Kansas City, Kansas 66160; ‡Program in Infectious Diseases, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104; and §Department of Medicine and Laboratory Medicine, University of Washington, School of Medicine, Seattle, Washington 98195

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The reactivates of human sera with uninduced and phorbol ester (TPA)-induced human herpesvirus-8 (HHV-8)-infected BCBL-1 cells were examined by immunofluorescence assay (IFA) and by radioimmunoprecipitation reactions (RIP). The seroprevalence of HHV-8 infections is low in the United States general population and only low levels of HHV-8 antibodies were detected in the seropositive sera. In contrast, high levels of antibodies against HHV-8 lytic and latent antigens were detected by IFA in the sera from HIV^+ Kaposi's sarcoma (KS)-positive individuals. These sera recognized several proteins and glycoproteins from BCBL-1 cells in RIP reactions. Two types of antibody responses were detected in the sera from HIV^+ KS^- homosexual men. In majority of the sera with and without detectable HHV-8 DNA in the peripheral blood mononuclear cells (PBMC), significantly low levels of HHV-8 antibodies were detected by IFA. These sera recognized only a subset of HHV-8 proteins and glycoproteins in RIP reactions. In contrast, in a subgroup of sera from HIV^+ KS^- homosexual men, higher levels of IFA antibodies against HHV-8 lytic and latent antigens were detected. These sera also recognized several viral proteins and glycoproteins in RIP reactions. These results suggest that antibody response profiles to HHV-8 infection vary significantly and serologic assays to detect antibody responses to a panel of both lytic and latent antibodies may be required for maximum sensitivity. Screening of a cDNA library from TPA-induced BCBL-1 cells with an HIV^+ KS^+ serum identified cDNAs encoding 12 HHV-8 proteins. Further characterization of these HHV-8 proteins would define the HHV-8 antigens useful for seroepidemiological studies and in discriminating lytic, latent, past, and/or reactivation infections.

INTRODUCTION

The newly identified human herpesvirus-8 (HHV-8) [or Kaposi's sarcoma-associated herpesvirus (KSHV)] DNA has been detected in more than 95% of Kaposi's lesions studied, in both HIV-negative and HIV-positive individuals and in all clinical subtypes of KS (Ambroziak *et al.,* 1995; Chang *et al.,* 1994, 1996; Dupin *et al.,* 1995; Huang *et al.,* 1995; Staskus *et al.,* 1997). In addition, HHV-8 sequences have been detected in body cavity-based B-cell lymphomas (BCBL) and several B-cell lines have been established from the BCBL tumors (Cesarman *et al.,* 1995a,b; Renne *et al.,* 1996). The BC-1 and HBL-6 cell lines carry both HHV-8 and Epstein–Barr virus (EBV). Upon phorbol ester (TPA) induction, a high level of EBV was reactivated from these cells with only a small amount of HHV-8 DNA replication (Cesarman *et al.* 1995a,b,; Moore *et al.,* 1996a). BCBL-1 cells carry only HHV-8 and a lytic cycle can be induced by TPA (Renne *et al.,* 1996). Sequences of about 140 kb HHV-8 DNA encoding more than 80 complete open reading frames (ORFs) have been published recently (Neipel *et al.,* 1997; Russo *et al.,* 1996). These HHV-8 ORFs are identified as ORFs 4 to 75 by their homology to ORFs of herpesvirus saimiri (HVS), a simian herpesvirus. HHV-8 also encodes 19 unique ORFs designated with a prefix K (Neipel *et al.,* 1997; Russo *et al.,* 1996). BCBL-1 cells were used in immunofluorescence (IFA) and Western blot assays to measure the latent and lytic antibody responses to HHV-8 in human sera (Gao *et al.,* 1996a,b; Kedes *et al.,* 1996; Koelle *et al.,* 1997; Simpson *et al.,* 1996; Smith *et al.,* 1997). HHV-8 seroprevalence varies according to the country of origin, with a higher prevalence in Africa (53–90%) (Gao *et al.,* 1996a; Simpson *et al.,* 1996). However, there are discrepancies in the incidence of HHV-8 infection reported by these studies and only a little is known about the antigens recognized by the sera. Identification and characterization of HHV-8 proteins recognized by human sera are fundamental to a rational understanding of the biology of HHV-8, its role in KS and other human diseases, and for diagnostic purposes. As an initial step, we tested sera in immunofluorescence and immunoprecipitation reaction assays with uninduced and TPA-induced BCBL-1 cells. Sera from HIV^+ and KS^- individuals and a subgroup of HIV^+ KS^- indi-

[1] To whom correspondence and reprint requests should be addressed. Fax: 913-588-7295. E-mail: bchandra@kumc.edu.

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TABLE 1

Reactivity of Human Sera in Immunofluorescence Assay with HHV-8-Infected BCBL-1 Cells

Sera from	No. tested	No. positive with TPA-induced[a] cells (%)	GMT (range)[b]	No. positive with uninduced cells (%)	GMT (range)	No. with PCR-positive PBMCs (%)
HIV^+ KS^+	44	43 (98)	449 (40–10,240)[c]	41 (93)	105 (40–1,280)[e]	27 (61)
HIV^+ KS^-	47	28 (60)	84 (40–1,280)[c]	16 (34)[g]	57 (40–320)[e]	12 (26)
Low seropositive		23	55 (40–160)[d]	11	40 (40–80)[f]	9
High seropositive		5	484 (320–1,280)	5	159 (80–320)	3
Healthy adults	120	9 (8)	53 (40–160)	4 (3)	47 (40–80)	ND
Children (ages 1–5 years)	110	0	—	0	—	ND

Note. Sera were tested for HHV-8 reactivity with uninduced and TPA-induced BCBL-1 cells by IFA. Specific reactivity at ≥1:40 dilution was considered positive for IFA. ND, not done.

[a] Phorbol ester.

[b] Geometric mean titer.

[c–f] Comparison of titers of anti-HHV-8 antibodies between groups was performed on log-transformed titers using Wilcoxon two-sample test and Kruskal–Wallis test.

[c] HIV^+ KS^+ vs HIV^+ KS^- lytic antibody response; $P = 0.0001$.

[d] HIV^+ KS^+ vs low seropositive HIV^+ KS^- lytic antibody response; $P = 0.0001$.

[e] HIV^+ KS^+ vs HIV^+ KS^- latent antibody response; $P = 0.0001$.

[f] HIV^+ KS^+ vs low seropositive HIV^+ KS^- latent antibody response; $P = 0014$.

[g] All sera positive for LANA type fluorescence with uninduced cells were also positive for lytic antibodies. Sera reactive only with uninduced cells were not detected.

viduals showed higher levels of antibodies against HHV-8 lytic and latent antigens. These sera immunoprecipitated several HHV-8-specific polypeptides and glycoproteins. The intensity of polypeptide bands increased with IFA titer. In contrast, most of the HIV^+ KS^- patients showed significantly low HHV-8 antibody titers, with and without HHV-8 DNA in their peripheral blood, and recognized only a subset of HHV-8 proteins. Screening of a cDNA library from TPA-induced BCBL-1 cells with an HIV^+ KS^+ serum identified cDNAs encoding 12 HHV-8 proteins.

RESULTS

HIV^+ KS^+ and HIV^+ KS^- sera show two types of HHV-8 antibody responses in IFA

All sera were tested by IFA with uninduced and TPA-induced BCBL-1 (HHV-8+) cells, with TPA-induced BJAB cells, and with TPA-induced P3HR-1 EBV producer cells. Specific granular punctate nuclear fluorescence with the majority (>80%) of uninduced BCBL-1 cells and bright cytoplasmic fluorescence with about 30% of TPA-induced BCBI-1 cells were considered as recognizing latent and lytic HHV-8 antigens, respectively (Kedes *et al.,* 1996; Lennette *et al.,* 1996; Smith *et al.,* 1997). About 3 to 8% of cells also showed moderate cytoplasmic fluorescence and to determine whether this was due to the reactivity with lytic cycle HHV-8 antigens, expression of lytic cycle HHV-8 proteins ORFs 22, 25, and 26 was examined by RT–PCR. The uninduced BCBL-1 cells were positive for these three ORFs and a higher quantity was detected from TPA-induced cells (data not shown). These results suggested that like P3HR-1 EBV producer cells and B95-8 (Kieff, 1996; Rickinson and Kieff, 1996), the BCBL-1 cell is a producer cell line with a low percentage of uninduced cells spontaneously producing lytic antigens.

Though sera were tested with a starting dilution of 1:10, several sera showed nonspecific fluorescence at 1:10 and 1:20 dilutions with BCBL-1 and BJAB cell. Hence, sera showing a reactivity at a dilution of 1:40 and above were considered positive and all positive sera were retested at least three times. Several sera from HIV^+ KS^- individuals, HIV^- healthy adults, and healthy children did not react with uninduced and TPA-induced BCBL-1 cells. This suggested that the reactivities of HIV^+ KS^+ sera were not against TPA-induced host cell proteins and were directed against HHV-8 lytic cycle-associated proteins induced by TPA. Ninety percent of sera from children and healthy adult men were positive for EBV-VCA antibodies and their titers ranged from 1:80 to 1:10,240, with a geometric mean titer (GMT) of 1:977. In contrast, none of the 110 sera from children (age 1–5) were positive for HHV-8 antibodies. Among the 120 sera from age-matched HIV^- healthy adult men tested, only 9 (8%) were positive for HHV-8 lytic antibodies and 4 of these 9 sera (3.3%) were also positive for antibodies against latent antigens and exhibited LANA type fluorescence. The antibody titers were also low, with GMTs of 53 and 47 for lytic and latent antibodies, respectively (Table 1). In contrast, the HHV-8 antibody titers from 44 HIV^+ KS^+ individuals with uninduced and induced BCBL-1 cells ranged from 1:40 to 1:10,240, with GMTs of 1:105 and 1:449, respectively (Table 1). When PBMC from these patients was examined, HHV-8 DNA was detected

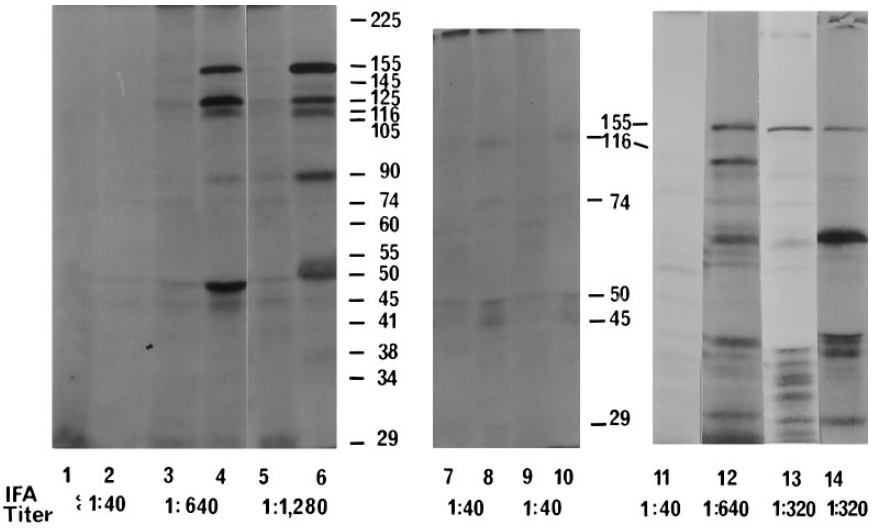

FIG. 1. Reactivities of human sera with HHV-8-infected uninduced and TPA-induced BCBL-1 cells in RIP reactions. Uninduced BCBL-1 cells (lanes 1, 3, 5, 7, and 9) and TPA-induced BCBL-1 cells (lanes 2, 4, 6, 8, 10, and 11–14) were labeled with [^{35}S]methionine and cysteine and lysates were reacted with sera. Lanes 1 and 2: HIV$^-$ and KS$^-$ and HHV-8 antibody-negative serum. Lanes 3–6: HIV$^+$ KS$^+$ individuals' sera. Lanes 7–11: HIV$^+$ KS$^-$ sera with low HHV-8 antibodies. Lanes 12–14: HIV$^+$ KS$^-$ sera with high HHV-8 antibodies. Samples were analyzed on 9% acrylamide cross-linked with *N-N'*-diallyltartardiamide. Standard molecular weight markers were included in parallel lanes. Numbers indicate approximate molecular mass (kDa) of HHV-8 polypeptides immunoprecipitated. IFA titer indicates antibody titer of sera as measured in an immunofluorescence assay with acetone-fixed TPA-induced HHV-8$^+$ BCBL-1 cells.

in 27 samples (27/44, 61%). Absorption with TPA-induced EBV producer cells (P3HR-1) did not alter the reactivities of these sera (data not shown), thus suggesting no interference due to cross-reacting antibodies under the conditions of our assays. Furthermore, though all KS patients were also positive for EBV-VCA, with antibody titers ranging from 1:80 to 1:10,240 (GMT, 786), there was no correlation between EBV-VCA and HHV-8 titers.

Among the 47 HIV$^+$ KS$^-$ gay men examined, 12 were positive for HHV-8 DNA in their PBMC (12/47, 26%), 28 of 47 (60%) were positive for lytic antibodies, and 16 of 47 (34%) sera were seropositive for LANA type IFA antibodies. All 16 sera positives for LANA type fluorescence were also positive for lytic antibodies. The overall geometric mean antibody titers against latent and lytic antigens (1:57 and 1:84) detected in these sera were significantly lower than the GMT detected in HIV$^+$ KS$^+$ patients ($P = 0.0001$, Wilcoxon two-sample test. Table 1). Interestingly, two types of antibody responses were detected in these HIV$^+$ KS$^-$ patients. Most of the sera (23/28, 82%) showed low levels of HHV-8 antibodies with uninduced (GMT, 40) and induced BCBL-1 cells (GMT, 55), which was significantly lower than the GMT detected in HIV$^+$ KS$^+$ patients ($P = 0.0014$ and $P = 0.0001$, respectively. Table 1). PBMC from 9 patients in this group (9/23, 39%) were positive for HHV-8 DNA. Another group of 5 HIV$^+$ KS$^-$ patients (5/28, 18%) showed higher levels of HHV-8 lytic antibodies (>1:320) and PBMC from 3 (3/5, 60%) were positive for HHV-8 DNA by PCR. The lytic HHV-8 antibody titers for the 5 patients were 1:320, 1:1280, 1:640, 1:640, and 1:640, respectively, with twofold lower antibody titers for LANA type fluorescence (Table 1). There were no statistical differences when the GMT against latent and lytic antigens detected in the 5 HIV$^+$ KS$^-$ high HHV-8 antibody sera was compared with the GMT detected in HIV$^+$ KS$^+$ patients (Table 1). The 23 HIV$^+$ KS$^-$ patients with low HHV-8 antibodies had a mean CD4$^+$ T-cell level of 326 (range 116–697) and the 5 HIV$^+$ KS$^-$ patients with high HHV-8 antibodies had a mean CD4$^+$ T-cell level of 130 (range 49–295).

Serological responses against HHV-8 are directed against a number of HHV-8 proteins

To define the HHV-8 proteins recognized by the above human sera, RIP reactions with uninduced and TPA-induced BCBL-1 cells labeled with [^{35}S]methionine + cysteine (3 to 4 days postinduction) were done. A composite of representative examples is shown in Fig. 1. A polypeptide of about 49 kDa was recognized weakly by all sera from uninduced and TPA-induced BCBL-1 (Fig. 1) and D6 cells (data not shown). This was considered nonspecific or due to cross-reacting antibodies. Besides this reaction, sera from HIV$^+$ and HIV$^-$ KS$^-$ adults negative for HHV-8 antibodies by IFA did not show any specific reaction by immunoprecipitation (Fig. 1, lanes 1 and 2). From TPA-induced cells, sera from HIV$^+$ KS$^+$ individuals recognized at least 20 polypeptides (Fig. 1, lanes 3–6) and among these, polypeptides with approximate molecular weights of 155, 145, 125, 116, 105, 90, 74, 60, 55, 50, 45, 41, 38, 34, and 29 kDa were prominent (Fig. 1, lanes 4 and 6). Only a weak reaction was seen with uninduced BCBL-1 cells and a limited number of polypeptides (225, 145, 125, 116, 105, 74, 70, 55, and 29

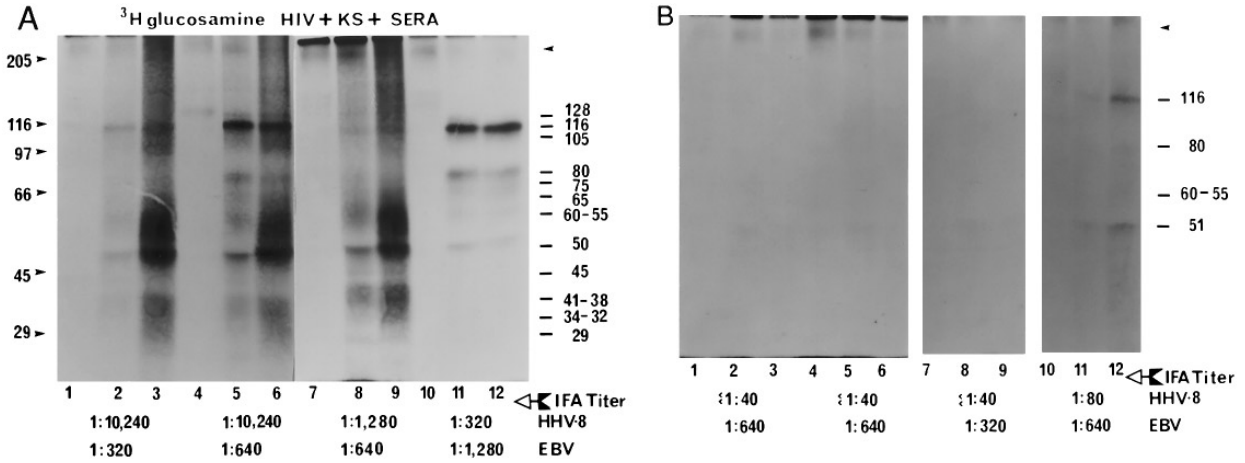

FIG. 2. (A and B) Reactivities of sera with HHV-8 glycoproteins. Cells were labeled with [^{3}H]glucosamine. Lanes 1, 4, 7, and 10: uninduced HHV-8-negative D6 cells. Lanes 2, 5, 8, and 11: uninduced BCBL-1 cells. Lanes 3, 6, 9, and 12: TPA-induced BCBL-1 cells. The arrow indicates the 235-kDa glycoprotein from all three cell lysates immunoprecipitated by all sera, irrespective of their HHV-8 antibody status. (A) Lanes 1 to 9: HIV$^+$ KS$^+$ sera. Lanes 10–12: HIV$^+$ KS$^-$ serum with high HHV-8 antibodies. (B) Lanes 1 to 9: HIV$^+$ KS$^-$ and HHV-8 antibody-negative sera. Lanes 10–12: HIV$^+$ KS$^-$ serum with low HHV-8 antibodies. Samples were analyzed on 9% acrylamide cross-linked with *N-N′*-diallyltartardiamide. Standard molecular size markers were included in parallel lanes. Numbers indicate approximate molecular weights (kDa) of HHV-8 polypeptides immunoprecipitated. IFA titer indicates antibody titer of sera as measured in an immunofluorescence assay with acetone-fixed TPA-induced HHV-8$^+$ BCBL-1 cells and with TPA-induced EBV producer P3HR-1 cells.

kDa) were immunoprecipitated (Fig. 1, lanes 3 and 5). Similar to these HIV$^+$ KS$^+$ sera, the five sera from HIV$^+$ KS$^-$ individuals with higher HHV-8 IFA antibody titers recognized at least 20 polypeptides from induced cells (Fig. 1, lanes 12 to 14). Absorption with TPA-induced EBV producer cells (P3HR-1) did not alter the reactivities of these sera (data not shown), thus suggesting no interference due to cross-reacting antibodies under the conditions of our assays. In contrast, sera from HIV$^+$ KS$^-$ individuals with HHV-8 in their PBMC and with low reactivity in IFA (titer 1:40) recognized only a subset of HHV-8 proteins with approximate molecular weights of 116, 74, 50, 45, and 29 kDa (Fig. 1, lanes 7 to 11). Similar reactions were also seen with sera from HIV$^-$ and KS$^-$ adult men with low HHV-8 antibody titers (data not shown). These bands were visible only after long exposure of the autoradiographs. The 155-kDa polypeptide was recognized only by sera with an IFA titer of $>$1:320.

Analysis of HHV-8 glycoproteins immunoprecipitated by human sera

Since glycoproteins of herpesviruses were shown to be good targets for human serological responses, we tested the human sera with [^{3}H]glucosamine-labeled uninduced D6 (HHV-8$^-$), uninduced, and TPA-induced BCBL-1 (HHV-8$^+$) cells. Equal amounts of TCA-precipitable counts (5×10^5 cpm) of cell lysates were used and representative results are shown in Figs. 2A and 2B. No specific reactivity was seen with HHV-8-negative D6 cells (Figs. 2A and 2B, lanes 1, 4, 7, and 10) and the reactivity with a high molecular weight polypeptide recognized by all HHV-8$^+$ and HHV-8$^-$ sera (Figs. 2A and 2B, arrow) was considered nonspecific. Similar reactions were seen with HHV-8$^-$ BJAB cells (data not shown). A barely detectable glycopolypeptide of about 49 kDa was recognized by all sera from uninduced and TPA-induced cells and was considered nonspecific or due to cross-reacting antibodies. Besides these minimal reactivities, HIV$^+$ HHV-8$^-$ sera did not show any specific reactivity with BCBL-1 cells (Fig. 2B, lanes 1–9). In contrast, HIV$^+$ KS$^+$ sera (Fig. 2A, lanes 1–9) and HIV$^+$ KS$^-$ sera with high HHV-8 antibodies (Fig. 2A, lanes 10–12) immunoprecipitated glycopolypeptides of 128, 116, 105, 80, 75, 65, 60–55, 50, 45, 41–38, 34–32, and 29 kDa from uninduced (Fig. 2A, lanes 2, 5, 8, and 11) and TPA-induced (Fig. 2A, lanes 3, 6, 9, and 12) BCBL-1 cells. The intensity of glycopolypeptide bands increased with IFA titer. Though equal TCA counts were used for these immunoprecipitation reactions, the intensity of bands recognized from induced BCBL-1 cells was much higher than that from bands from uninduced BCBL-1 cells, suggesting increased synthesis of these glycopolypeptides after TPA induction.

In contrast to these results, HIV$^+$ and HIV$^-$, KS$^-$ low HHV-8 antibody titer sera ($<$1:160) weakly recognized the glycopolypeptides of 116, 80, 60–55, and 51 kDa (Fig. 2B, lane 12). Autoradiographs shown here were exposed for 4 weeks and the glycoproteins immunoprecipitated by these sera were barely detected even after 8 weeks of exposure. Experiments with inhibitors of glycosylation and glycosidic enzymes indicate the presence of sugar residues in these proteins (data not shown), eliminating the possibility of incorporation of radiolabels into these proteins after the metabolic breakdown of the [^{3}H]glu-

TABLE 2

HHV-8 ORFs Encoded by cDNAs Identified by an HIV^+ KS^+ Serum[a]

HHV-8 ORFs encoded by cDNAs	Amino acids in the full-length genomic ORFs[c]	Predicted molecular weight (kDa) encoded by the full-length ORFs[c]
6	1133	130
8	845	94
9	1012	113
25	1376	153
26	305	34
39	401	45
59	396	43
65	170	18
68	545	60
73	1162	135
K8.1A[b]	228	25
K8.1B[b]	167	19

[a] The cDNA library in ZAPII was constructed by using mRNA isolated from TPA-induced BCBL-1 cells. This library was screened with an HIV^+ KS^+ serum. Immunoreactive phages were picked and purified by four subsequent steps of screening. The identified cDNAs were released into the plasmid forms, sequenced, and compared with the sequences in the data bank.

[b] ORFs encoded by spliced messages.

[c] From Russo *et al.* (1996) and Neipal *et al.* (1997).

cosamine. The ability of sera to immunoprecipitate glycoproteins from BCBL-1 cells was not related to EBV VCA titers, since many sera with high EBV VCA titers did not recognize HHV-8 proteins (Fig. 2B, lanes 1–9). The reactivities of human sera with the HHV-8 glycoproteins suggested that HHV-8 glycoproteins may serve as good target antigens for the development of sensitive and highly specific serological assays.

Identification of genes encoding immunogenic proteins of HHV-8

To identify the genes encoding immunogenic HHV-8 proteins, a serum from HIV^+ KS^+ individual with high HHV-8 antibodies (1:10,240) in IFA was used to screen the cDNA library from induced BCBL. After four screenings, 56 cDNA clones were isolated and released in plasmid forms by cocultivation with the helper phage. Overlapping clones of these cDNAs (both orientations) were sequenced. Sequences were analyzed for ORFs and, comparison with published genomic HHV-8 sequence identified 12 ORFs. These are: ORFs 6, 8, 9, 25, 26, 39, 59, 65, 68, 73, K8.1A, and K8.1B and the predicted molecular weights of proteins encoded by the full-length ORFs are given in Table 2. The putative functions for some of these ORFs are designated by comparison with other herpes virus ORFs and these are ORF6, ssDNA binding protein; ORF-8, glycoprotein gB; ORF-9, DNA polymerase; ORF25, major capsid protein; ORF26, minor capsid protein; ORF59, DNA replication protein; ORF65, capsid protein (Lin *et al.,* 1997; Simpson *et al.,* 1996); and ORF73, putative LANA antigen (Rainbow *et al.,* 1997). All these cDNA-encoded proteins were in-frame with the 35-aa β-galactosidase fusion protein, thus suggesting specific reactivity with HHV-8 ORFs.

The complete ORF 65 is encoded by the cDNAs and in the other ORF cDNAs the length of ORFs ranged from 280 to 800 amino acids. However, identification of protein in the cDNA by HIV^+ KS^+ serum suggests that they encode the immunogenic part. ORF 65 is identified by other studies as immunogenic, which was recognized by about 85% of HIV^+ KS^+ sera (Lin *et al.,* 1997; Simpson *et al.,* 1996). ORF 73 is identified as responsible for the majority of the LANA type reactivities of sera (Rainbow *et al.,* 1997). Four cDNA clones in the above screening encode for a novel HHV-8 glycoprotein, ORF K8.1. The genomic ORF 8.1 is 197 aa long, with a N-terminal signal sequence and without any transmembrane sequence. However, comparisons of two full-length cDNA sequences show ORFs encoded by spliced messages (B. Chandran *et al.,* manuscript in preparation). One cDNA encodes for an ORF designated K8.1A, 228 aa long, a typical class I glycoprotein with a cleavable signal sequence(s), a transmembrane domain (TM), and four putative N-glycosylation sites (N). The splicing event has generated the transmembrane domain not seen in the genomic ORF. Another cDNA encodes K8.1B, an ORF of 167 aa, sharing similar amino and carboxy termini with K8.1A ORF. The splice acceptor sites for both cDNAs are the same; however, the donor site for K8.1B cDNA is upstream of the splice donor site in K8.1A cDNA. This has resulted in an in-frame deletion (B. Chandran *et al.,* manuscript in preparation).

DISCUSSION

To measure the human antibody responses against HHV-8 antigens associated with latent and lytic replicative cycles, BCBL cells carrying only HHV-8 or both HHV-8 and EBV were used in IFA and Western blot assays (Gao *et al.,* 1996a,b; Kedes *et al.,* 1996; Koelle *et al.,* 1997; Simpson *et al.,* 1996; Smith *et al.,* 1997). These studies suggest that HHV-8 seroprevalence in the normal population varies according to the country of origin, reflecting the differences in incidence of classical KS. Our studies and others demonstrate that the seroprevalence of HHV-8 infections is low in the United States general population and only low levels of HHV-8 antibodies were detected. However, there are discrepancies in the incidence of HHV-8 infection reported by these studies and only a little is known about the antigens recognized by the sera.

Higher HHV-8 seropositivity was reported among HIV^+ KS^- and HIV^- KS^- individuals in a study by Lennette *et al.,* (1996). This study also used TPA-induced BCBL-1 cells in IFA and 96% of AIDS–KS patients from the United

States and 100% of classic patients from Africa were positive for lytic antigens. Only 52% of United States AIDS–KS patients were positive for latent antigens, which is lower than the 81–83% positivity for United States AIDS–KS reported by Kedes *et al.* (1996) and Gao *et al.* (1996a, b). In contrast, 100% of African AIDS–KS patients were positive for latent antigens. HHV-8 seropositivity rates reached 90–100% in HIV-infected homosexual cohorts from the San Francisco area, which is higher than reported by others and by us. In addition, seroprevalence in the normal adult population was reported to be about 20–30%. These sera were tested only at a 1:10 dilution and the titers were not reported. In the recent studies by Simpson *et al.* (1996) and Lin *et al.* (1997) using HHV-8 ORF 65, 84% of sera from KS patients recognized ORF 65 protein and 82% reacted with LANA. About 30% of HIV^+ KS^- homosexual men, 3–5% of blood bank donors from the United States and the United Kingdom, and 53% of HIV^+ and HIV^- sera from Uganda were positive by these two assays. The reason for the discrepancies in the serological studies is not clear and maybe due to the different assay systems used and the populations studied. Lennette *et al.* (1996) tested the seroprevalence in the general population only at a 1:10 dilution of the sera. In our studies, though sera were tested with a starting dilution of 1:10, several sera showed nonspecific fluorescence at 1:10 and 1:20 dilutions with BCBL-1 and BJAB cells. These nonspecific reactions were seen even using the mouse anti-human monoclonal antibodies as described by Lennette *et al.* (1996) (data not shown). Hence, we considered sera showing a reactivity at 1:40 and above positive. In our studies, all sera positives for lytic antibodies were also positive for latent antibodies and no sera exhibiting only LANA type fluorescence were detected. Detection of a higher percentage of lytic HHV-8 antibodies than the antibodies against latent antigens by IFA (Table 1) suggests that detecting LANA type fluorescence may be specific yet may not be sensitive, and seroepidemiological studies should include measurement of both types of antibody responses.

The Simpson *et al.* (1996) and Lin *et al.* (1997) studies identified HHV-8 ORF 65 as an immunogenic lytic cycle-associated protein. However, not all HIV^+ KS^+ sera showed positive reaction with the ORF 65 protein, suggesting that an immunodominant antigen needs to be used. As shown in our studies, humoral responses are directed against a number of HHV-8 proteins, and a majority of HIV^+ KS^- individuals have low titers of HHV-8 antibodies, recognizing only a subset of viral proteins. Hence it is critical to use antigens detecting the persisting antibody responses for studies of seroprevalence. HHV-8 proteins identified here represent soluble proteins enriched by our detergent extraction procedure and many insoluble proteins may have been removed from the antigen preparation. The use of Protein A agarose precludes the detection of any polypeptides recognized by IgM antibodies. Similarly, BCBL-1 cells collected after 4 days of TPA induction used in these studies may overrepresent viral structural proteins and HHV-8 polypeptides detected in these studies may not fully represent HHV-8 early viral proteins. Hence, the number of polypeptides identified here represents a minimum estimate for the number of HHV-8 proteins eliciting antibody response in humans. Despite these limitations, our studies show that at least 20 electrophoretically distinct polypeptides were immunoprecipitated by the sera from HHV-8-infected cells, but not from uninfected cell extracts. These reactivities show that antibody responses against HHV-8 are directed against a variety of HHV-8-specific proteins, as in other herpesviruses. Gao *et al.* (1996b) used the BC-1 cell line ($HHV8^+$, EBV^+) in a Western blot assay and identified a 234/226 kDa doublet protein associated with the nuclei of uninduced cells as a HHV-8-specific latency-associated protein in their serological assay. In the RIP reactions, a 225-kDa polypeptide was observed from uninduced cells (Fig. 2, lanes 3, 4, 5, 14) and could represent the 234/226 kDa protein observed by Gao *et al.* (1996b). A weaker reaction in RIP reactions could be due to poor solubility of this protein.

Our data also indicate differential responses against different HHV-8 antigens. For example, all HIV^+ KS^+ sera and the five HIV^+ KS^- sera with higher HHV-8 antibodies recognized the 155- kDa polypeptide, whereas none of the HIV^+ KS^- sera with low titer HHV-8 antibodies did (Fig. 1). Similarly, HIV^+ KS^+ $HHV\text{-}8^+$ individuals recognized at least 12 HHV-8-specific glycoproteins and among these, only the glycoproteins of 110, 80, 60–55, and 50 kDa were very weakly recognized by HIV^+ KS^- sera (Fig. 2B). This suggests that responses against a specific subset of HHV-8 proteins may be short-lived and may rise during active viral replication. For example, reactivity of a serum with only 110- and 50-kDa glycoproteins but not with the 55-, 38-, and 34-kDa glycoproteins may be suggestive of past infection. Reactivity with all these glycoproteins would suggest recent and/or reactivation infection. Identification of genes encoding these HHV-8 proteins and examining large numbers of sera from HIV^+ KS^+ and HIV^+ KS^- $HHV\text{-}8^+$ individuals with these cloned and expressed genes would define the HHV-8 antigens that can discriminate past or present or reactivation infection. The partial list of genes identified here is an initial step toward these goals. The number of cDNAs recognized by an HIV^+ KS^+ serum here represents a minimum estimate for the number of HHV-8 proteins eliciting antibody response in humans. ORFs 65 and 73 were identified by other investigators as immunogenic lytic and latent antigens, respectively (Simpson *et al.,* 1996; Rainbow *et al.,* 1997). Direct comparison of predicted molecular weights of proteins encoded by the full-length ORFs (Table 2) with the molecular weights of proteins recognized by human sera is not possible due

to the posttranslational modifications of proteins *in vivo.* Further characterization of these HHV-8 proteins would define the HHV-8 antigens useful for seroepidemiological studies and in discriminating lytic, latent, past, and/or reactivation infections. In ongoing studies, K8.1A ORF (glycoprotein) expressed as a GST fusion protein was tested in Western blot assays. The results show that the antibodies against K8.1A ORF persist in HHV-8-infected individuals and testing the antibodies by this ORF provides a very sensitive and specific HHV-8 serological assay (B. Chandran *et al.,* manuscript in preparation).

High levels of antibodies against HHV-8 lytic and latent antigens in HIV^+ KS^+ patients and low levels of antibodies in the majority of HIV^+ KS^- individuals were detected in our studies. The limited number of polypeptides recognized by the majority of HIV^+ KS^- sera suggests that responses against these proteins may be persisting. The apparent quantity of HHV-8 proteins and glycoprotein immunoprecipitated by the sera correlated with the increase in IFA titer, which may be indicative of possible stimulation of antibody responses in these individuals. Stimulation may occur either due to viral replication (reactivation infection) or due to polyclonal stimulation. Since HHV-8 was isolated from the cell-free saliva of some of the HIV^+ KS^+ patients tested in the present study (Koelle *et al.,* 1997), this suggests that reactivation could attribute to the detection of higher HHV-8 antibody titer ($>$1:320). Using Gardella gel analyzes, Decker *et al.* (1996) also have detected linear forms of HHV-8 viral genome in PBMC from KS patients, an indication of HHV-8 viral replication in these patients. Studies including ours (Cesarman *et al.,* 1996; Staskus *et al.,* 1997; Smith *et al.,* 1997) have also detected messages encoding HHV-8 lytic antigens in KS lesions. A subset of HIV^+ individuals reported here showed higher levels of HHV-8 lytic antibodies and three (3/5) were positive for HHV-8 DNA. This group could represent patients with HHV-8 lytic replication.

The relationship between the detection of increased levels of HHV-8 antibodies and AIDS–KS is not clear at present. The Gao *et al.* (1996a,b) studies show that 21 of the 40 HIV^+ patients seroconverted to LANA type antibodies 6 to 75 months before the clinical appearance of KS, suggesting that the rise in antibodies against LANA may also suggest an increase in latently infected endothelial cells, B cells, and other cells and/or increase in the transformed cells expressing latency-associated proteins. These patients were not tested for lytic antibody responses. Our studies have so far not detected low lytic antibody responses and high LANA type latency-associated antibodies. Taken together, these data suggest that the development of KS may be associated with HHV-8 reactivation and detection of higher levels of lytic and/or latent antibody responses may indicate tumor development and/or progression. In another study, 71% of HIV patients with KS were shedding HHV-8 in saliva and showed statistically significant higher HHV-8 lytic antibodies than the HIV patients without KS (Koelle *et al.,* 1997). HHV-8 was detected in the saliva of only 15% of HIV patients without KS. The amounts of HHV-8 DNA were 2–4 logs lower than those detected in HIV^+ KS^+ patients' saliva. This also supports the theory that reactivation of HHV-8 may precede the development of clinical KS.

Since the 23 HIV^+ KS^- patients with low HHV-8 antibodies have a mean $CD4^+$ T-cell level of 326 (range 116–697) and the 5 HIV^+ KS^- patients with high HHV-8 antibodies have a mean $CD4^+$ T-cell level of 130 (range 49–295), this suggested that the detection of higher levels of HHV-8 antibodies in the 5 patients may not be due to the immunocompetent status of these individuals. However, for a meaningful correlation and to determine whether immunosuppression is a potential confounder, HIV^+ patients with low and high HHV-8 antibodies described here need to be monitored sequentially for the levels of HHV-8 antibodies, HHV-8 DNA, $CD4^+$ T-cell levels, HIV levels, and for the development of KS; such prospective studies are currently in progress. HHV-8 reactivation probably occurs under reduced immunosurveillence conditions as a result of ongoing HIV infection. Regression of KS after withdrawal of immunosuppressive therapy in transplantation individuals (Safai and Schwartz, 1992) shows the important role of the immune system in controlling KS and possibly HHV-8. Recent reports show that foscarnet and ganciclovir may have some activity in preventing KS (Mocroft *et al.,* 1996). Since these drugs are effective against lytic virus replication, this also indirectly suggests that HHV-8 lytic replication may have a role in KS and may precede KS development. However, a better understanding of the serological responses against HHV-8 latent and lytic antigens and their correlation with KS development and progression is critical. If HHV-8 lytic replication indeed precedes the development of KS, then the measurement of lytic antibody responses could serve as a diagnostic and/or prognostic marker for tumor development and/or regression and might have important implications in the assessment of therapeutic intervention of lytic HHV-8 infection.

MATERIALS AND METHODS

Cell lines

$HHV\text{-}8^-$ and EBV^- body cavity B-cell lymphoma cell line BCBL-1 (Renne *et al.,* 1996), $HHV\text{-}8^+$ and EBV^+ HBL-6 cells (Moore *et al.,* 1996a), EBV producer cell line P3HR-1, and EBV^- B-cell line BJAB were grown in RPMI 1640 medium with glutaMAX I (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. The BCBL-1 cells were a gift from Dr. McGrath (UCLA) and the HBL-6 cells were a gifts from Dr. P. Moore (Columbia University, NY). Single cell clones

from HBL-6 cells (HHV-8$^+$, EBV$^+$) were established by limiting dilutions and were tested by PCR for HHV-8 and EBV DNA using primers as described before (Smith *et al.,* 1997) and by IFA for EBV and HHV-8 using human sera (Smith *et al.,* 1997). Three types of clones were obtained: HHV-8$^+$, EBV$^+$, HHV-8$^-$ EBV$^+$; and HHV-8$^-$, EBV$^-$. These were recloned and retested. The cell lines designated D6 and C6 (HHV-8$^-$ and EBV$^-$) were used as controls for the experiments described here.

DNA extraction

Details of collection of DNA from peripheral blood specimens, primers amplifying HHV-8 DNA sequences, primers amplifying regions internal to the outer primers, and sequences of internal probes used for Southern blot hybridization were as described before (Koelle *et al.,* 1997; Smith *et al.,* 1997). Several precautions were taken to avoid contamination in PCR reactions. Extractions were done in a location having no cloned or amplified materials corresponding to the genes detected by PCR. Cells were extracted for DNA in a laboratory in another building, using materials dedicated to this purpose, and PCR reactions were set up using filter tips and separate materials in a different laboratory from that used for analysis of cloned and amplified materials.

Serum samples

Forty-four sera from HIV$^+$ KS$^+$ adult men patients, 47 sera from HIV$^+$ KS$^-$ patients (homosexual men), 120 sera from age-matched HIV$^-$ and KS$^-$ adult men, and 110 sera from children (ages 1 to 5) were used in these studies. All sera were stored at −20°C and heat inactivated at 56°C for 30 min before use.

Immunofluorescence assay (IFA)

IFA was done according to procedures described before (Smith *et al.,* 1997). Briefly, 10^7 cells in 10 ml medium were induced with 20 ng/ml of phorbol ester (TPA; Sigma, St. Louis, MO). Uninduced and TPA-induced BCBL-1 cells, TPA-induced P3HR-1 cells, and TPA-induced BJAB cells were collected, washed in phosphate-buffered saline, pH 7.4 (PBS), spotted on slides (5 mm inner diameter, 10 circles per slide), air dried in a laminar flow hood under UV light, and fixed in cold acetone for 10 min. Fixed cells were incubated for 30 min at 37°C with twofold dilutions of human sera beginning at 1:10. After incubation, slides were washed rigorously in PBS. The slides were then incubated for 30 min at 37°C with a prestandardized dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (Hyclone, Logan, UT). After washing, the slides were counterstained with a 1:20,000 dilution of Evans blue (Sigma) for 5 min at room temperature, washed, and mounted with 50% (vol/vol) glycerol in PBS. Slides were examined under a fluorescence microscope.

Radiolabeling, radioimmunoprecipitation (RIP), and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Uninduced and TPA-induced (72–96 h postinduction) cells (10^7) were labeled for 20 h with 250 μCi of [^{35}S]methionine and cysteine (Tran^{35}S-label, sp act, 1177 Ci/mmol; ICN, Irvine, CA) or with 500 μCi of [^{3}H]glucosamine (sp act, 25 Ci/mmol; American Radiolabeled Chemicals Inc., St. Louis) in deficient DMEM (Sigma). Immunoprecipitation was carried out according to procedures described before (Balachandran *et al.,* 1989; Chang and Balachandran, 1991; Smith *et al.,* 1997). Briefly, cells were solubilized with lysing buffer (0.05 M Tris–hydrochloride, pH 7.5, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 100 U of aprotinin/ml, 0.1 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged at 100,000 *g* for 1 h. Equal trichloroacetic acid-precipitable counts (5×10^5 cpm) of cell lysates were mixed with 10 μl of antibodies and 100 μl of Protein-A–Sepharose beads (Pharmacia; Piscataway, NJ) and were kept rocking at 4°C for 2 h. The precipitates were washed, dissociated by boiling in sample buffer, and analyzed by SDS–PAGE in 9–12% acrylamide cross-linked with 0.28% *N, N′*-diallyltartardiamide (DATD; Sigma). Molecular weight markers (Sigma) were electrophoresed in parallel channels. Gels were stained, destained, infused with 1 M salicyclic acid, dried on filter paper, and placed in contact with XAR-5 film (Kodak; Rochester, NY) at −70°C for fluorography.

Construction of cDNA library and screening of lambda ZAPII expression system

Total RNA extracted from TPA-induced BCBL-1 cells (24, 48, 72, and 96 h postinduction) were treated with DNase and verified for the absence of HHV-8 DNA by DNA-PCR using ORF 22, 25, and 26 primers (Smith *et al.,* 1997). The mRNA was isolated by oligotex mRNA isolation kit (Qiagen, Chatsworth, CA) and the purified mRNAs were used in cDNA synthesis using oligo(dT) linker–primer containing a *Xho*I site (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Synthesized cDNAs were tested for HHV-8 messages by PCR for HHV-8 ORFs 22, 25, and 26 using primers described before (Smith *et al.,* 1997) and appropriate size fragments were detected. The cDNA library in the Lambda ZAPII expression system was made according to the manufacturer's recommendations. After amplification, this library had a titer of about 4×10^9 PFU with 99% recombinant phages. This library was screened with a HIV$^+$ KS$^+$ serum using procedures described before (Chang and Balachandran, 1991). To remove nonspecific reactivities, the serum was absorbed with HHV-8$^-$ control B cells (D6 and BJAB) and with bacterial lysates with nonrecombinant phages. Appropriate dilution of the antibody was used to screen the library. Immunoreactive

phages were picked and purified by four subsequent steps of screening. After four screenings, 56 clones were isolated. The identified cDNAs were released into the plasmid forms by *in vivo* excision using the ExAssist helper phage.

DNA sequence analysis

The cDNA clones (both orientations) were sequenced in the Biotechnology Center at the University of Kansas Medical Center. Sequences were analyzed and compared with the data bank by the BLAST program.

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